Mouse Myeloma Proteins with Antihapten Antibody Activity. The Protein Produced by Plasma Cell Tumor MOPC-315*

Herman N. Eisen, Ernest S. Simms, and Michael Potter

ABSTRACT: The myeloma proteins produced by 116 transplantable mouse plasma cell tumors were screened for antibody activity to the 2,4-dinitrophenyl and 2,4,6-trinitrophenyl groups. Seven active proteins (six immunoglobulin A's and one immunoglobulin H) were identified. The most active one, an immunoglobulin A myeloma protein, was produced by tumor MOPC-315. Except for its remarkable homogeneity with respect to affinity (intrinsic association constant), this protein reacted with 2,4-dinitrophenyl and 2,4,6-trinitrophenyl ligands in essentially the same way as the anti-2,4-dinitrophenyl and anti-2,4,6-trinitrophenyl antibodies formed in response to conventional immunization procedures. As isolated from serum, protein 315 was polydisperse and precipitated specifically with 2,4-dinitrophenyl and 2,4,6-trinitrophenyl proteins. A 7S monomer, derived by reduction of the polydisperse material, and a proteolytic ("Fab") fraction, obtained by papain digestion of the 7S monomer, no longer precipitated with 2,4-dinitrophenyl and 2,4,6-trinitrophenyl proteins, but were otherwise fully reactive with 2,4-dinitrophenyl and 2,4,6-trinitrophenyl ligands, *e.g.*, bound ligands quenched the proteins tryptophan fluorescence and underwent characteristic red shifts in absorption spectra. Equilibrium dialysis and fluorescence quenching titrations showed the 7S monomer and Fab fraction to have, per 120,000 and 55,000 molecular weight, respectively, a single homogeneous binding site of high affinity for ε-2,4-dinitrophenyl-L-lysine (intrinsic association constants at 4° were 1.6×10^7 and 1.2×10^7 M^{-1}, respectively). It is not clear whether 315 and the other myeloma proteins with antibody activity are constitutive (*i.e.*, formed without intervention of an antigen) or induced, or if induced by what immunogen.

By whatever process they arise, further study of this group of proteins ought to provide insight into the structural basis for the specificity of antibody molecules in general.

The recent finding of a human myeloma protein with antibody activity for ε-DNP-L-lysine (Eisen *et al.*, 1967a) prompted us to search for similar immunoglobulins among those produced by transplantable plasma cell tumors of mice. Of 74 mouse myeloma sera examined by the same spectral screening method employed previously with human sera, four reacted positively and one (MOPC-315) reacted especially well. Since sera from mice bearing tumor 315 also formed specific precipitates with DNP and with TNP proteins, these and other mouse myeloma sera were examined by gel diffusion reactions. Tested all together, by one or both assays, there were 116 transplantable tumors, producing 3 IgM, 66 IgA, 14 IgF, 12 IgG, and 21 IgH myeloma proteins. Seven myeloma proteins (six IgA and one IgH) reacted with DNP, TNP, or both ligands. Three of the seven precipitated with DNP and TNP proteins (MOPC-315, MOPC-460 and MOPC-292), one precipitated with TNP but not with DNP proteins (MOPC-378), and three precipitated with neither (MOPC-329, MOPC-228, and MOPC-379, which was an IgH protein). Some properties of the most strongly reacting protein (315)[1] are described here; some of the other reactive proteins will be described in later papers.

Materials and Methods

Induction of the Tumor and Purification of the Myeloma Protein. Tumor MOPC-315 was induced in a heterozygous (seven backcross generations) BALB/c-2 mouse (Potter and Lieberman, 1967a,b) by three intraperitoneal injections of 0.5 ml of Bayol F (Potter and Boyce, 1962), given at 2, 4, and 6 months of age. In BALB/c-2 the immunoglobulin heavy-chain linkage group from the C57BL/6 strain is introgressively backcrossed onto BALB/c. A plasma cell tumor arising in such an animal expresses an immunoglobulin heavy-chain gene from only one of the two chromosomes with the heavy-chain linkage group (Warner *et al.*, 1966). The IgA immunoglobulin made by MOPC-315 carries the A^{12} determinant (Lieberman and Potter, 1966) of the heavy-chain linkage group of BALB/c. MOPC-315 does not produce

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[1] Terminology: Nomenclature and abbreviations for immunoglobulins correspond to those recommended by the World Health Organization (*Bull. World Health Organ. 30*, 447 (1964)). The plasma cell tumor is referred to as MOPC-315, and the immunoglobulin it produces as protein 315, or simply 315.

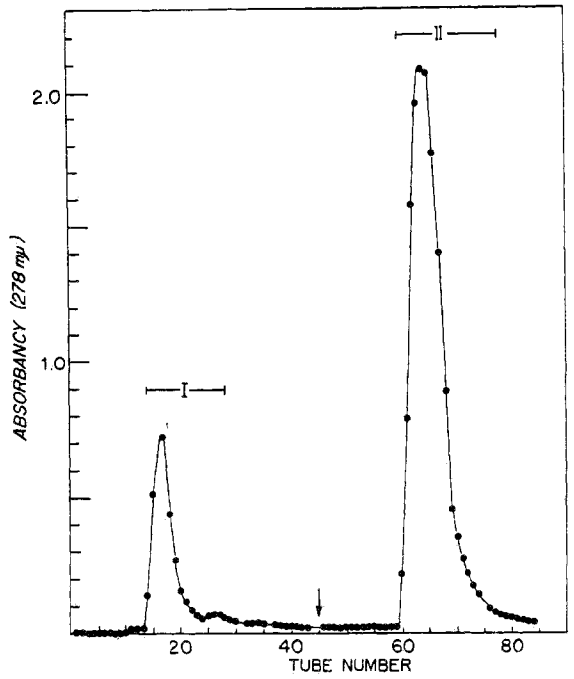

FIGURE 1: Isolation of protein 315. To 5.0 ml of pooled serum from mice carrying tumor MOPC-315 (14th transplanation generation) were added 5.0 ml of buffered saline[2] and 6.67 ml of cold, saturated $(NH_4)_2SO_4$. After 15 min at 0°, the precipitate was collected, dissolved in 5.0 ml of water, dialyzed against buffered saline, and added to a 20-g (dry weight) column of DEAE-Sephadex A-25 (40–120 μ), equilibrated with buffered saline (packed bed was 2 × 25 cm; flow rate 4–4.5 ml/min; 2.5 ml/tube). At tube 45 (arrow) the developing buffer was changed to 0.3 M NaCl–0.1 M potassium phosphate (pH 8.0). Fraction I did not precipitate with DNP-HSA and by fluorescence quenching its activity was marginal (13% quenching by ε-DNP-L-lysine at 1×10^{-6} M). Fraction II was highly active by both assays (see text). Applied to the column were 67.5 A_{278} units; recovery was 11.2 A_{278} units in fraction I and 44.7 A_{278} units in fraction II.

a Bence–Jones protein. The mouse in which this tumor arose had not been intentionally immunized with any known antigen. The tumor was transplanted to fresh BALB/c hosts at 3-week intervals, at which time donors were exsanguinated. Sera were pooled and stored at −20°.

Myeloma protein 315 was isolated from serum by precipitation with $(NH_4)_2SO_4$, followed by chromatography on DEAE-Sephadex (Figure 1). The active fraction (fraction II) was polydisperse, with approximately 7S, 9S, 11S, and 13S components in the ultracentrifuge, as with many other mouse IgA myeloma proteins (Fahey, 1961; Potter and Kuff, 1961; Lieberman *et al.*, 1968). Mild reduction with 0.005 M dithiothreitol (2 hr, pH 8, room temperature), followed by alkylation with a threefold molar excess of iodoacetamide (0°, 30 min, pH 8.2) converted the polydisperse material completely into a single sedimenting form ($s_{20,w}$ = 6.6 S), hereafter called the 7S monomer. The 7S monomer was purified further, in about 50% yield, by immunoadsorption on DNP-HSA[2]

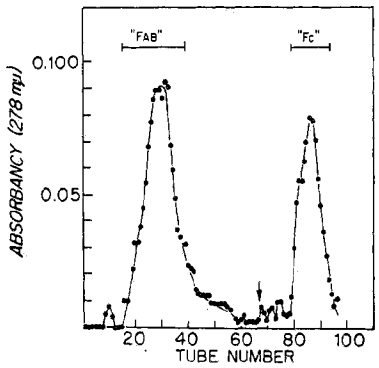

FIGURE 2: Separation of proteolytic fragments of 315. The 7S monomer, purified by immunoadsorption, was digested with papain (added at 1.5% the weight of the protein), dialyzed against 0.05 M NaCl–0.01 M potassium phosphate (pH 7.9), and added to a column of DEAE-Sephadex A-25 (packed bed, 2 × 20 cm; flow rate, 1.0 ml/min; 3.5 ml/tube). Tubes 1–66 were eluted with a linear gradient 0.05–0.3 M NaCl in 0.01 M potassium phosphate (pH 7.9). At tube 66 (arrow) the developing buffer was changed to 0.3 M NaCl–0.1 M potassium phosphate (pH 7.9). Added to the column were 7.1 A_{278} units (88% of the 7S monomer subjected to digestion), and essentially all were recovered. (Heterogeneity in the Fab fraction was indicated by differences in fluorescence quenching titrations of selected tubes; *e.g.*, tryptophan fluorescence of the earlier tubes was quenched slightly more by ε-DNP-L-lysine than were later tubes. This fraction was further purified by immunoadsorption.)

which had been coupled to bromoacetylcellulose (Robbins *et al.*, 1967), followed by elution with 0.1 M DNP-glycine (pH 8.2). The eluate was passed through Dowex (1-X8, 20–50 mesh) to remove DNP-glycine, or through a mixed column with a bed of DEAE-cellulose above Dowex 1-X8 in order to remove traces of soluble DNP-HSA, as well as DNP-glycine (Eisen *et al.*, 1967b).

Papain Digestion. After 75-min digestion at 37° with papain in 0.01 M cysteine at pH 7.4 (Porter, 1959), the 7S monomer was degraded completely to fragments that sedimented at $s_{20,w}$ = 3.5 S. The digest (80–90%)[3] was retained after dialysis against buffered saline. Chromatography on DEAE-Sephadex A-25 separated the digest into two fractions (Figure 2). The first, called Fab because it had the same ligand binding activity as the whole molecule and contained light-chain peptides (see below), amounted to 65% of the dialyzed digest. About 78% of this fraction was taken up by the immunoadsorbant, and 64% of the absorbed material was recovered after elution with 0.1 M DNP-glycine. In addition, when the entire dialyzed digest was treated directly with the immunoadsorbant, 60% was specifically adsorbed. Thus the active fraction in the papain digest amounted to about 43–50% of the 7S monomer (*e.g.*, 0.60 × 0.85), in agreement with an estimate of 45% based on molecular weight values (55,000 for Fab *vs.* 120,000 for the 7S monomer; see below).

[2] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: dansyl, 5-dimethylaminonaphthalenesulfonyl; HSA, human serum albumin; BγG, bovine γ-globulin; *t*-Boc, *t*-butyloxycarbonyl; buffered saline, 0.15 M NaCl–0.01 M potassium phosphate (pH 7.2–7.4); DNFB, 2,4-dinitrofluorobenzene.

[3] Absorbancy at 278 mμ was used to follow the distribution of protein and protein fragments through purification and fractionation procedures.

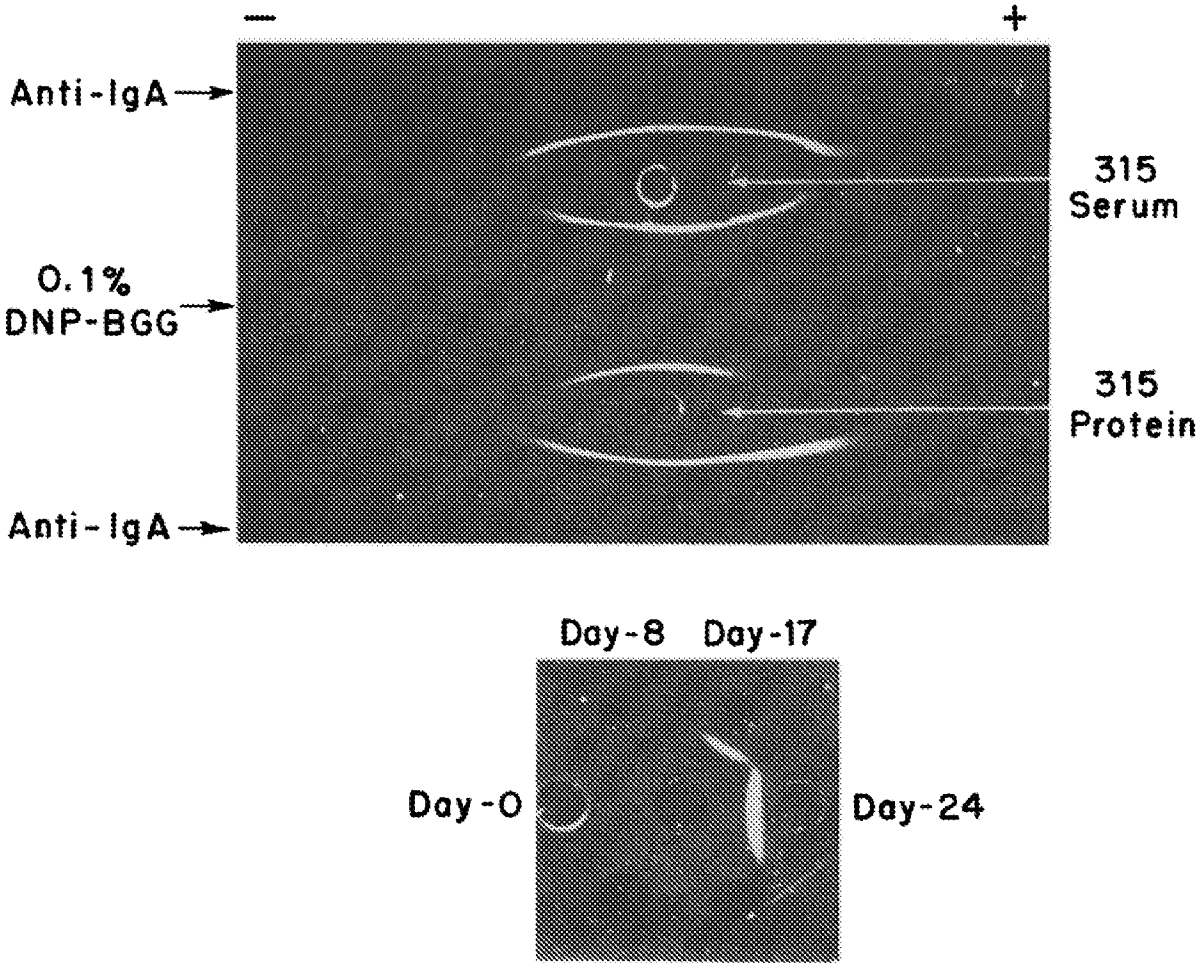

FIGURE 3: Agar gel studies. upper: Agar gel immunoelectrophoresis (Potter and Kuff, 1961) of serum from mice bearing MPOC-315 and of isolated protein 315 (fraction II, Figure 1). The buffer was 0.05 M Tris-acetate (pH 8.2). lower: Agar gel diffusion (Ouchterlony) precipitin reactions of serum samples taken serially from the same mouse, into which MOPC-315 had been transplanted. Day 0, before transplantation; day 8, small tumor growth; days 17 and 24, large tumor growth. The appearance of precipitating activity with DNP-BγG coincides with progressive tumor growth.

The second fraction eluted from DEAE-Sephadex, called Fc, amounted to about 35% of the dialyzed digest (Figure 2). Quenching of its tryptophan fluorescence by ε-DNP-L-lysine was marginal (0–20% in different preparations), and was probably due to contamination by the Fab fraction.

Separation of Light and Heavy Chains. Protein 315 was extensively reduced with 0.01 M dithiothreitol in 1.0 M Tris-HCl (pH 8.2), and 7.0 M guanidine hydrochloride at 37° for 1 hr, and was then alkylated with 0.022 M iodoacetamide at 4° for 15 min. Light and heavy chains were separated on a Sephadex G-100 column (2.4 × 100 cm), equilibrated, and developed with 6 M urea, 0.01 M acetic acid, and 0.0037 M 2-mercaptoethanol (Potter *et al.*, 1964). Tryptic peptide maps of the separated chains were prepared as previously described (Katz *et al.*, 1959; Potter *et al.*, 1964).

Extinction Coefficients and Molecular Weights. With crystallized bovine serum albumin as a standard ($E^{1\,cm}_{0.1\%}$ 0.67 at 278 mμ), extinction coefficients were determined in a Spinco Model E ultracentrifuge from the area under the boundary formed in a synthetic boundary cell, under conditions where each sample formed a single boundary. The values obtained (absorbancy at 278 mμ/(mg/ml), 1-cm light path) were 1.40 for fraction II, 1.25 for the 7S monomer, and approximately 1.40 for the Fab fraction. The value for fraction II agreed with that determined by Kjeldahl analysis (1.42), assuming 16% N.[4]

The number-average molecular weight for the 7S monomer was determined in a Mechrolab membrane osmometer (protein concentrations 1.6–5.6 mg/ml); by this method $\bar{M}_n$ = 117,300.[4] By sedimentation equilibrium with protein concentrations from about 80 to 400 μg per ml, the weight-average molecular weight (Yphantis, 1964) was 113,400 or 122,700, assuming partial specific volumes of 0.720 and 0.740, respectively.[4] For purposes of calculation, a provisional molecular weight

[4] We are grateful to Dr. Jacqueline Reynolds for molecular weight determinations by membrane osmometry, to Miss Carmelita Lowry and Dr. Maria Michaelides for the sedimentation equilibrium values, and to Dr. S. Frankel for the Kjeldahl analysis.

value of 120,000 for the 7S monomer was used. For the Fab fraction a tentative value of 56,500 was obtained by membrane osmometry, and 55,000 was used for calculations. Molecular weights of these materials and of heavy and light chains are being examined further in a separate study.

Equilibrium Dialysis. In small lucite chambers[5] maintained at 4° without agitation, 50 μl of protein solution was separated by dialysis membranes from 50-μl samples of [^{3}H]ε-DNP-L-lysine at various concentrations. After concentration equilibrium was reached (about 40 hr), 25-μl samples from the compartments with and without protein were counted in Bray's (1960) solution in a Packard scintillation spectrometer. Quenching corrections (made by recounting after addition of a [^{3}H]-toluene standard) were negligible. Of the added ^{3}H, 82–96% was recovered from all chambers.

[^{3}H]ε-DNP-L-Lysine. [^{3}H]DNFB (Nuclear, Chicago; 10.4 mCi/μmole) and nonradioactive DNFB, added as carrier, were reacted for 18 hr at 43° with a fivefold excess of α-*t*-Boc-L-lysine (Fox Chemical Co., Los Angeles) in 0.05 M potassium phosphate (pH 7.4). After removal of the *t*-Boc group with 1 N HCl in glacial acetic acid, the mixture was dried, taken up in water, extracted with peroxide-free ether, and chromatographed on thin-layer silica gel with water-saturated methyl ethyl ketone as solvent. The yellow zone whose R_F matched that of authentic ε-DNP-L-lysine (R_F about 0.2) was removed and eluted with water or dilute ethanol.

Purity of the labeled DNP-lysine was established by mixing it with the authentic compound and dialyzing to equilibrium against an equal volume (1.0 ml) of a solution of purified rabbit IgG anti-DNP antibody. Measured by absorbance at 360 mμ and ^{3}H counting, the millimicromoles of authentic ^{1}H and labeled [^{3}H]ε-DNP-L-lysine bound per millimicromole of antibody were 1.74 and 1.72, respectively. The labeled and unlabeled haptens also had identical absorption spectra (λ_{max} 363 mμ; A_{363}/A_{400} = 2.0), and were indistinguishable as ligands in fluorescence quenching titrations (see Figure 7).

Other Methods and Reagents. Previously described procedures and materials were used for precipitin reactions in agar gel (Dray *et al.*, 1963) and in buffered saline (Eisen, 1964), fluorescence quenching titrations (Velick *et al.*, 1960; Eisen, 1964), obtaining difference absorption spectra between bound and free ligands (Little and Eisen, 1967a), and preparing DNP and TNP proteins, in which almost all lysine residues were substituted (Little and Eisen, 1967b).

Results

Precipitin Reactions. In gel diffusion precipitin tests, sera from mice carrying MOPC-315 formed precipitates with DNP-Bγ G, DNP-HSA, TNP-Bγ G, and TNP-HSA,

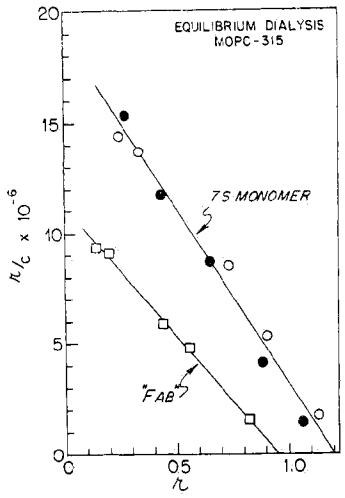

FIGURE 4: Equilibrium dialysis. The 7S monomer was 2.4 μg in 0.05 ml (○) or 2.0 μg in 0.05 ml (●); the latter value was corrected for 15% contamination because the protein had not been purified by immunoadsorption. Specific activity of [^{3}H]ε-DNP-L-lysine was 67,530 cpm/mμmole (○, □) or 94,020 cpm/mμmole (●). Conditions: 4°; 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4). Each point is the average of duplicate chambers, which agreed to within ±5%.

but not with unsubstituted Bγ G or HSA, or with the following conjugates: *p*-iodophenylsulfonyl-HSA,[6] *p*-toluenesulfonyl-HSA,[6] dansyl-HSA,[7] 8-anilinonaphthalene-1-sulfonate-5-azo-BSA,[7] 8-azonaphthalene-1-sulfonate-Bγ G,[7] fluorescein-HSA,[7] and tetramethylrhodamine-HSA.[7]

The reactive component in sera of mice bearing MOPC-315 was precipitated by a specific rabbit antiserum to mouse IgA (Lieberman and Potter, 1966) and distributed electrophoretically both anodically and cathodically at pH 8.2 (Figure 3). This heterogeneity is probably due to the polydispersity of protein 315 in serum (see Methods). Serum of normal BALB/c mice contains no precipitins for DNP or TNP proteins detectable in gel diffusion tests. Upon transplantation and progressive growth of MOPC-315 in BALB/c mice, precipitins for DNP proteins appeared in increasing concentration (Figure 3). The tumor is currently in its 25th transplantation generation and continues to produce the DNP-reactive protein in each new recipient. By day 21–24 after transplantation, when recipients have large tumors (3–5 g), pooled serum has usually contained per ml about 7 mg of protein precipitable by DNP-Bγ G (see below).

The precipitin reaction with DNP–Bγ G in liquid (phosphate buffered saline) showed classic zones of antibody excess, equivalence, and antigen excess, and washed specific precipitates could be completely dis-

[5] The chambers are a modification of those obtainable from Technilab Instruments, Los Angeles, Calif. Details for their construction and use may be obtained from the Department of Microbiology, Washington University School of Medicine, St. Louis, Mo.

[6] Kindly provided by Dr. Richard Asofsky, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Md.

[7] Kindly provided by Dr. Charles W. Parker, Washington University School of Medicine, St. Louis, Mo. These protein conjugates had from about 7 to 20 moles of substituent groups per gram molecular weight of protein.

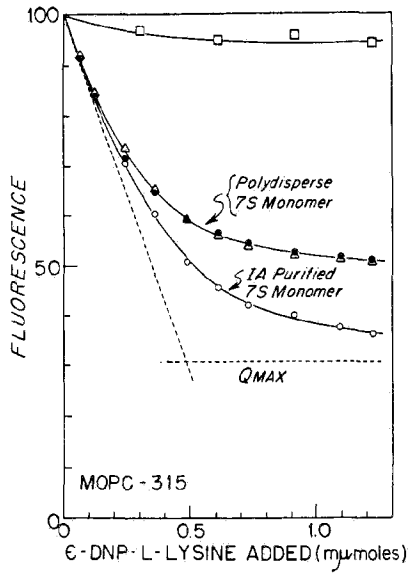

FIGURE 5: Fluorescence quenching titrations. The polydisperse protein of fraction II (see Figure 1) (●), the 7S monomer produced from it (△), the immunoadsorbant- (IA) purified 7S monomer (○), and the inactive material (in the crude 7S) not bound by the immunoadsorbant (□) were each titrated at 4° with 6.1×10^{-6} M ε-DNP-L-lysine in 0.15 M NaCl–0.01 M potassium phosphate (pH 7.4). Excitation at 295 mμ; fluorescence emission recorded at 345 mμ. The Q_{max} value (69% quenching of the initial fluorescence) is that of the IA-purified 7S monomer, of which 0.43 mμmole was titrated; the equivalent amount of ε-DNP-L-lysine was 0.48 ± 0.06 mμmole (intersection of Q_{max} with the linear extrapolation of the initial slope). The Q_{max} value was obtained in a separate titration with 4×10^{-4} M ε-DNP-L-lysine, correcting nonspecific attenuation of fluorescence by a parallel titration of tryptophan, as the free amino acid (McGuigan and Eisen, 1968).

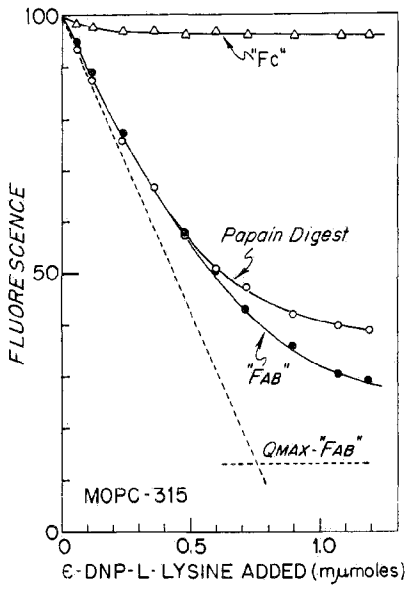

FIGURE 6: Fluorescence quenching titrations of a dialyzed papain digest of the 7S monomer (○) and the fractions resolved from it by chromatography plus immunoadsorption (Fab fraction, ●; Fc fraction, △). With the Fab, 0.79 mμmole was titrated and the equivalent amount of ε-DNP-L-lysine was 0.8 ± 0.08 mμmole (intersection of linear extrapolation of initial slope to Q_{max}). The Q_{max} value was obtained in separate titrations of the Fab fraction with 4×10^{-4} M ε-DNP-L-lysine (see legend of Figure 6). All titrations were under conditions given in Figure 6.

solved within a few minutes when suspended in excess hapten, *e.g.*, 0.1 M DNP-glycine.

The precipitin reaction was also inhibited by univalent DNP-haptens; *e.g.*, with ε-DNP-L-lysine at a total concentration of 0.45, 1.8, 7.1, and 28×10^{-5} M the amount of protein 315 precipitated from 0.05 ml of serum (by 65 μg of DNP-BγG) was 326, 294, 187, and 36.4 μg, respectively, as compared with 380 μg in the absence of the inhibitor. The amounts of precipitated protein 315, expressed as a proportion of the amount precipitated in the absence of inhibitor, were not linear with total hapten concentration. This result, which was also obtained with fraction II (Figure 1), implies that some molecules of polydisperse 315 were more refractory than others to hapten inhibition of precipitation (Pauling *et al.*, 1944). Since the combining sites of protein 315 were homogeneous with respect to intrinsic association constant (see below, and Figures 4 and 7), it is likely that higher multimers, with more combining sites per molecule, have a smaller probability of being fully inhibited than lower multimers. This explanation could be tested if the diverse multimers can be isolated as stable, individual components.

The polydisperse protein isolated as fraction II was 84–87% precipitable by DNP-HSA at equivalence, but the 7S monomer was not precipitated at all with DNP or TNP proteins. This result is in accord with the univalency of the 7S monomer, determined by equilibrium dialysis and fluorescence quenching (see below).

Equilibrium Dialysis. The equilibrium dialysis data obtained with the 7S monomer and the active proteolytic (Fab) fragment are plotted in Figure 4 according to $r/c = nK - rK$, where r is moles of hapten bound per mole of protein, c is the concentration of free hapten, n is the number of combining sites per molecule of protein, and K is the intrinsic association constant.

Three findings are notable. (1) The plots are clearly linear (*e.g.*, coefficient of correlation for the linear regression of the 7S monomer is −0.976), showing that the protein's binding sites are homogeneous with respect to affinity for the hapten. (2) Affinity is high and nearly the same for the 7S monomer and the Fab fraction ($K = 1.6 \times 10^7$ and 1.2×10^7 M^{-1}, respectively). (3) The abscissa intercept, corresponding to n, is 1.2 for the 7S monomer; these data, as well as the fluorescence quenching titrations shown below are incompatible with 2.0 ligand binding sites/molecule. A 10% error in extinction coefficient or in the provisional molecular weight of 120,000 would bring the value for n close to 1.0. The Fab fraction is also univalent, with close to 1.0 site/55,000 molecular weight.

Equilibrium dialysis with the polydisperse fraction II (Figure 1) was unsatisfactory, because recovery of ligand (counts per minute) was erratic and low. We presume that, at the low protein concentrations required and the long time needed for equilibration, a substantial proportion of the protein in this form stuck to the dialy-

sis membrane or chamber. Binding of ligands by fraction II was, however, readily measured by fluorescence quenching titrations, which are completed in a few minutes.

Fluorescence Quenching. Figures 5 and 6 show that the tryptophan fluorescence of protein 315 was extensively quenched by ε-DNP-L-lysine; and almost identical titrations were obtained with ε-DNP-aminocaprocate and with ε-TNP aminocaproate.

The specificity of these reactions was evident from control titrations with ε-dansyl-L-lysine.[7] This ligand quenches the tryptophan fluorescence of antidansyl antibody and the bound ligand fluoresces at 500 mμ when this antibody–ligand complex is excited at 295 mμ (Parker *et al.*, 1967). When added at 5×10^{-5} M (an eightfold higher concentration than ε-DNP-L-lysine in Figure 5), ε-dansyl-L-lysine caused only marginal reduction in the tryptophan fluorescence of protein 315 (maximal decrease in fluorescence was 12%) and an identical effect was observed with a preparation of purified, high-affinity rabbit–anti-DNP antibody ($K_0 \geq 1 \times 10^8$ M^{-1} for ε-DNP-L-lysine at 4°). In addition, no enhancement of the dansyllysine fluorescence (at 500 mμ) was detected in the titration of protein 315. It is likely that the marginal effect on fluorescence of protein 315 and rabbit anti-DNP antibody was simply due to absorption by the high concentration of unbound dansyllysine.

There was a distinct difference in the titration of the 7S monomer before and after treatment with the immunoadsorbant, indicating that the polydisperse protein in fraction II (Figure 1), and the 7S monomer derived from it, contained impurities (before immunoadsorption). We estimate that the contaminants amounted to about 15% (based on absorbancy at 278 mμ), since only 85% of this 7S monomer was bound by the immunoadsorbant, and fluorescence of the unadsorbed material was not quenched by hapten (Figure 5). These results agree with the observation that only 85% of fraction II was precipitable by DNP-BγG and DNP-HSA (see Precipitin Reactions).

The Fab fraction had 85–90% of its tryptophan fluorescence quenched when saturated by hapten (Figure 6). A similarly high degree of quenching by bound ligand is observed with similar fragments from conventional high-affinity rabbit anti-DNP and anti-TNP molecules of the IgG class (Little and Eisen, 1968; McGuigan and Eisen, 1968).

Linear extrapolation of initial slopes of fluorescence quenching titrations to Q_{max} values indicated that both the immunoadsorbant-purified 7S monomer and the Fab fragment derived from it had close to 1.0 binding site per 120,000 and 55,000 molecular weight, respectively (Figures 5 and 6), in agreement with the equilibrium dialysis data of Figure 4.

As is shown in Figure 7, the intrinsic association constants determined by fluorescence quenching and by equilibrium dialysis were in agreement. The slopes of the logarithmic Sip's plots in Figure 7 (0.98 and 0.86 for the Fab fraction and 7S monomer, respectively) are also consistent with the equilibrium dialysis evidence for homogeneity of the combining sites (Figure 4) (*cf.* Eisen and Siskind, 1964; Fujio and Karush, 1966).

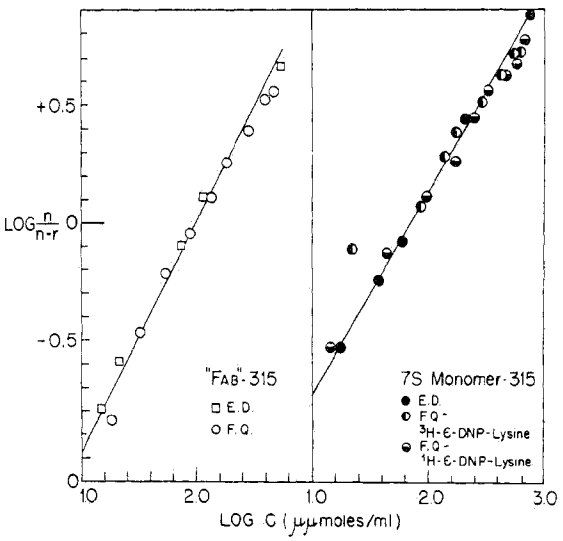

FIGURE 7: Comparison of binding data obtained by equilibrium dialysis and fluorescence quenching titration. Fab (left): equilibrium dialysis data (□) are from Figure 5; the fluorescence titration (○) was obtained with another preparation of Fab and [^{1}H]ε-DNP-L-lysine, and calculated with $n = 1.0$ and $Q_{max} = 85$. 7S monomer (right): equilibrium dialysis data (●) are from Figure 4 (closed circles), and fluorescence quenching was obtained with the same protein and either [^{3}H]- or [^{1}H]ε-DNP-L-lysine and calculated with $n = 1.2$ and $Q_{max} = 60$. All data were obtained at 4° in buffered saline. Heterogeneity index (slope) is 0.98 for Fab and 0.86 for 7S monomer.

Titrations of the polydisperse material of fraction II and of the 7S monomer derived from it were virtually superimposable (Figure 5), and their Q_{max} values were also the same. These results indicate that the active sites in multimers and in the 7S monomer of 315 had essentially the same affinity for ε-DNP-L-lysine. These results also suggest that the univalency and high affinity of this IgA monomer are not artifacts of mild reduction and alkylation. The properties of the "native" 7S monomer, isolated directly from serum rather than by reduction of multimers, will have to be studied.

Spectral Shifts. Figure 8 shows two peaks in the difference absorption spectrum between ε-DNP-L-lysine in buffered saline and in serum from mice carrying MOPC-315. With normal BALB/c serum, in contrast, the difference spectrum has no peaks over the wavelengths of interest (400–500 mμ) and is characterized only by a sloping, broad, negative deflection, due to binding of the hapten by serum albumin (Carsten and Eisen, 1953, and unpublished observations). With protein 315, the polydisperse fraction II (Figure 1), the 7S monomer, and the Fab fraction all gave the same two maxima at 470 and 383 mμ, which are essentially the same as those observed when ε-DNP-L-lysine is bound by the anti-DNP and anti-TNP antibodies raised by conventional immunization in the rabbit, guinea pig, goat, horse (Little and Eisen, 1967a,b), and chicken (E. W. Voss and H. N. Eisen, in preparation). The difference spectrum of ε-TNP-L-lysine also had a maximum at 470 mμ, but the second peak was at 373 mμ; *i.e.*, 10 mμ lower than with ε-DNP-L-lysine, and this also corresponds to what is ob-

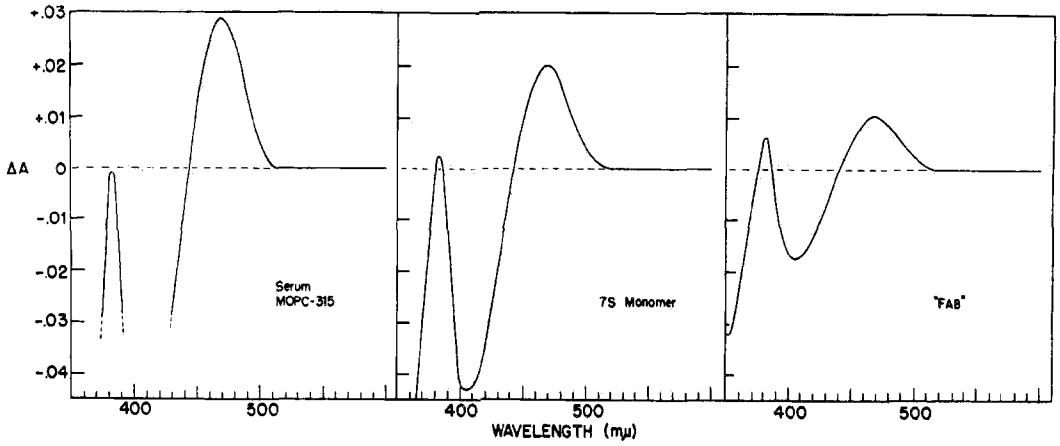

FIGURE 8: Change in absorption spectrum of ε-DNP-L-lysine bound to protein 315. The difference absorption spectra were obtained with ε-DNP-L-lysine at 2.3×10^{-5} M in either twofold-diluted serum (left), the 7S monomer at 1.34 mg/ml (middle), or the Fab at 0.45 mg/ml (right). All spectra were obtained at about 7° in buffered saline.

served when ε-TNP-L-lysine is bound by conventional anti-DNP and anti-TNP antibodies (Little and Eisen, 1967a).

The increase in molar absorptivity, $\Delta\epsilon_M$, of bound ε-DNP-L-lysine at 470 mμ was about 1400 for the 7S monomer and Fab (Figure 8). For protein 315 in whole serum $\Delta\epsilon_M \cong 900$; this value is probably too low because of the presence of serum albumin, which also binds this ligand and causes a hypochromic effect (Carsten and Eisen, 1953). These values for $\Delta\epsilon_M$ are fairly close to those obtained with ε-DNP-aminocaproate bound to a preparation of rabbit anti-DNP antibody ($\Delta\epsilon_M \cong 600$; Little and Eisen, 1967). They provide additional evidence that the combining site of protein 315 is like that of conventional anti-DNP and anti-TNP antibodies.

Tryptic Peptide Maps. The map of the heavy chain isolated from 315 resembled that of other BALB/c α chains. Common peptides, corresponding to the Fc region, as well as distinctive peptides, were recognized (Figure 9) (Lieberman *et al.*, 1968). On the basis of common peptides, the light chain was identified as κ (Figure 9). A large number of distinguishing peptides were also evident in the light-chain map (Potter *et al.*, 1964).

The Fab tryptic peptide map contained fewer distinct peptides than the maps of the isolated chains, and many of the Fab peptides corresponded to variable peptides of the light chains. However, a number of light-chain peptides were not evident, and these included the common κ peptides, which are usually demonstrated readily by this technic. While only few heavy-chain peptides were discernible in the Fab map, considerable material remained at the origin (Figure 9).

Discussion

The foregoing results show that the IgA myeloma protein produced by mouse plasma cell tumor MOPC-315 contains a remarkably homogeneous, high-affinity combining site, through which it reacts with DNP and TNP ligands in the same way as the antibodies produced in mammals and chickens immunized intensively with dinitrophenylated or trinitrophenylated immunogens. It precipitates with DNP and TNP proteins, binds DNP-lysine in equilibrium dialysis, and bound DNP and TNP ligands quench the protein's tryptophan fluorescence and undergo a characteristic red shift in absorption spectrum. Moreover, the ligand binding properties of the protein are accounted for, stoichiometrically and energetically, by a small proteolytic fraction (Fab) containing part of the light chain. The binding reactions of protein 315 seem specific since (1) over 90% of the other A-myeloma proteins tested were inactive, and (2) protein 315 did not react significantly with a number of other relatively hydrophobic benzenoid groups, *e.g.*, dansyl, anilinonaphthalenesulfonyl, azonaphthalenesulfonyl, and *p*-toluenesulfonyl.

The apparent univalency of the 7S monomer of protein 315 presents an interesting contrast to the well-established bivalency of IgG antibodies. While the number of combining sites of conventionally induced IgA antibodies has not yet been measured, we expect that their 7S monomers will, like that of protein 315, also prove to be univalent. Evidence from studies of IgM antibodies suggest that this type of structure may be more prevalent than was previously suspected. Most studies of IgM molecules show five combining sites per pentamer of 900,000 molecular weight or one per 7S monomer (Onoue *et al.*, 1965; Lindquist and Bauer, 1966; Metzger, 1967; Voss and Eisen, 1968; Clem and Small, 1968). However the number of sites is still uncertain because the low affinity of IgM antibodies for simple ligands, and their heterogeneity with respect to affinity, makes it difficult to establish a value with confidence.[8]

In contrast to the binding properties of protein 315, many of its structural features are not clear. Our pro-

[8] A recent study of an IgM antibody preparation with relatively high affinity for 1-azonaphthalene-8-sulfonate suggests that there may be, per 7S monomer, two combining sites, one with about 100-fold higher affinity than the second (K. Onoue, A. L. Grossberg, Y. Yagi, and D. Pressman, personal communication). If this result is generally valid, IgM antibodies would still differ fundamentally from IgG, whose two combining sites per molecule are almost certainly equivalent.

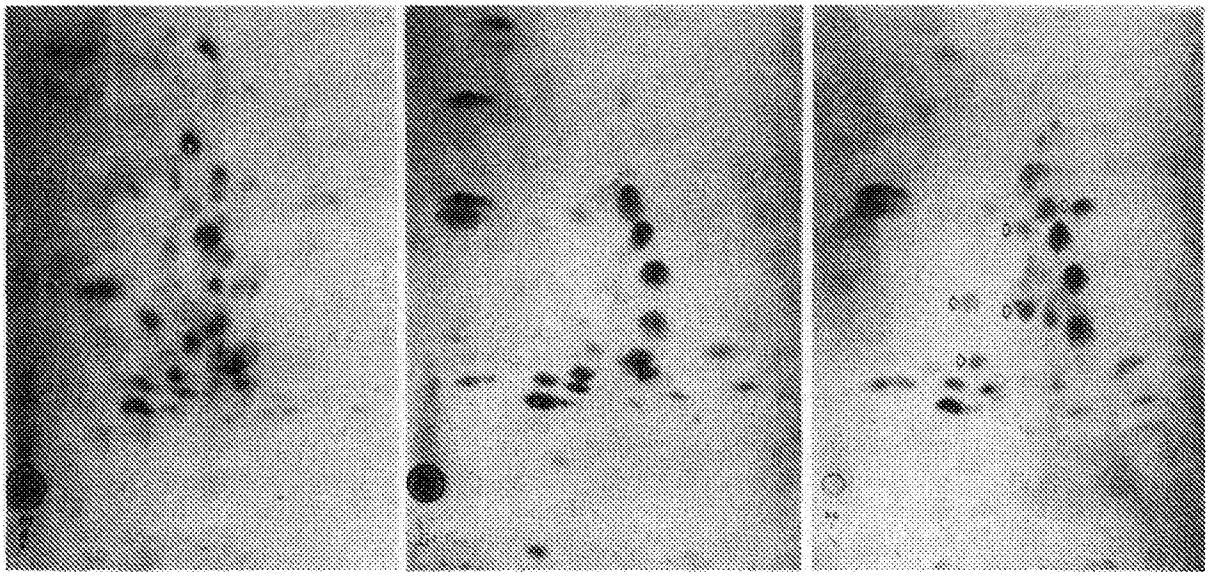

FIGURE 9: Tryptic peptide maps of 315 heavy chain (left), papain Fab (center), and light chain (right). The origin is at the lower left corner. Chromatography (left to right) in 1-butanol–acetic acid–water was run first, followed by electrophoresis at pH 3.6 in pyridine–acetic acid for 65 min at 3200 V. The arrows indicate the common κ-chain peptides (Potter *et al.*, 1964).

visional molecular weight of 120,000 is in accord with Abel and Grey (1968), whose preliminary findings indicate that mouse IgA myeloma proteins have relatively small α chains and a weight ratio of α:κ chains of about 2:1, which is consistent with a four-chain monomer. Since the purified Fab fraction, derived by proteolysis of the 7S monomer and immunoadsorption, corresponds to about 45% of the whole molecule, and also has one combining site with the same affinity as the monomer, it is possible that the cooperative interaction of segments of four chains (two from light and two from heavy chains) may be necessary to constitute the active site in protein 315. The tryptic peptide maps, which suggest that papain may cleave this IgA molecule in quite a different way than IgG molecules, could be in accord with this view. Obviously, far more extensive studies are required to evaluate this and other possibilities.

Perhaps the most unexpected aspect of this study has been the finding that about 5% of the mouse IgA myeloma proteins tested reacted with the DNP group. One possible interpretation of this extraordinarily high incidence is based on the notion that these proteins are constitutive; *i.e.*, they arise without intervention of an antigenic stimulus. For example, these proteins could represent a subclass of α or κ chains, or a particular combination of subclasses, in which a binding site for polynitrobenzenes is fortuitously formed from "constant" amino acid sequences peculiar to these subclasses. If this were true, all the proteins in this hypothetical subclass might be expected to have the same affinity for DNP or TNP ligands. However, preliminary findings with two other proteins of this group that are currently being studied (MOPC-460 with Dr. B. Jaffe and MOPC 329 with Miss E. Schulenberg) indicate that they differ in affinity from each other and from 315.

It was previously suggested that myeloma proteins of a given specificity, such as anti-DNP, might be more commonplace than is ordinarily suspected, if the Fab regions of each immunoglobulin were to contain a large number of different combining sites, each directed against a different ligand (Eisen *et al.*, 1967a). This speculation is not ruled out by the present findings, but it is not a likely explanation for them. If, for example, there were 10^4 noncross-reacting determinants, of which DNP and TNP were one, and each of the 10^4 were represented in a different set of 5% of mouse myeloma proteins, each of these proteins would have on the average 500 combining sites ($10^4 \times 0.05$), each of a different specificity.

Rather than being constitutive, these proteins could be produced by neoplastic clones that had been somehow selected by an unknown immunogen that is prevalent in BALB/*c* mice injected with mineral oil. It is unlikely that immunogenic DNP- or TNP-like contaminants are present in the Bayol F used to induce MOPC 315, since this oil is used in the preparation of Freund adjuvant mixtures for routine immunizations of many species of vertebrates; without the addition of appropriate immunogens these mixtures do not induce the formation of anti-DNP or -TNP antibodies, or prime animals for a secondary response (Steiner and Eisen, 1967).

Subsequent to the present observations, D. Schubert, A. Jobe, and M. Cohn (personal communication) found that a number of their mouse IgA myeloma proteins also precipitated with DNP-HSA, and that some of them in addition also precipitated with 5-acetouracil-BSA or with purin-6-oyl-BSA. Accordingly, they have suggested that nucleic acids, liberated in the inflammatory response to the oil and acting as immunogens, may have induced the formation of these proteins, whose binding of DNP ligands would then represent a cross-reaction. This would accord with evidence that macroglobulin paraproteins with antibody activity appear to be specific for "self-antigens" (*e.g.*, Stone and Metzger, 1967). If these proteins are produced as a result of induction with a "self-antigen," myeloma proteins of other spe-

cificities ought to be inducible with conventional foreign antigens. Whatever the mechanism for their origin may be, the present group of proteins, each of which is probably homogeneous in primary structure and has a distinctive affinity for simple DNP or TNP ligands, is likely to be of value for exploring the structural basis for antibody specificity.

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